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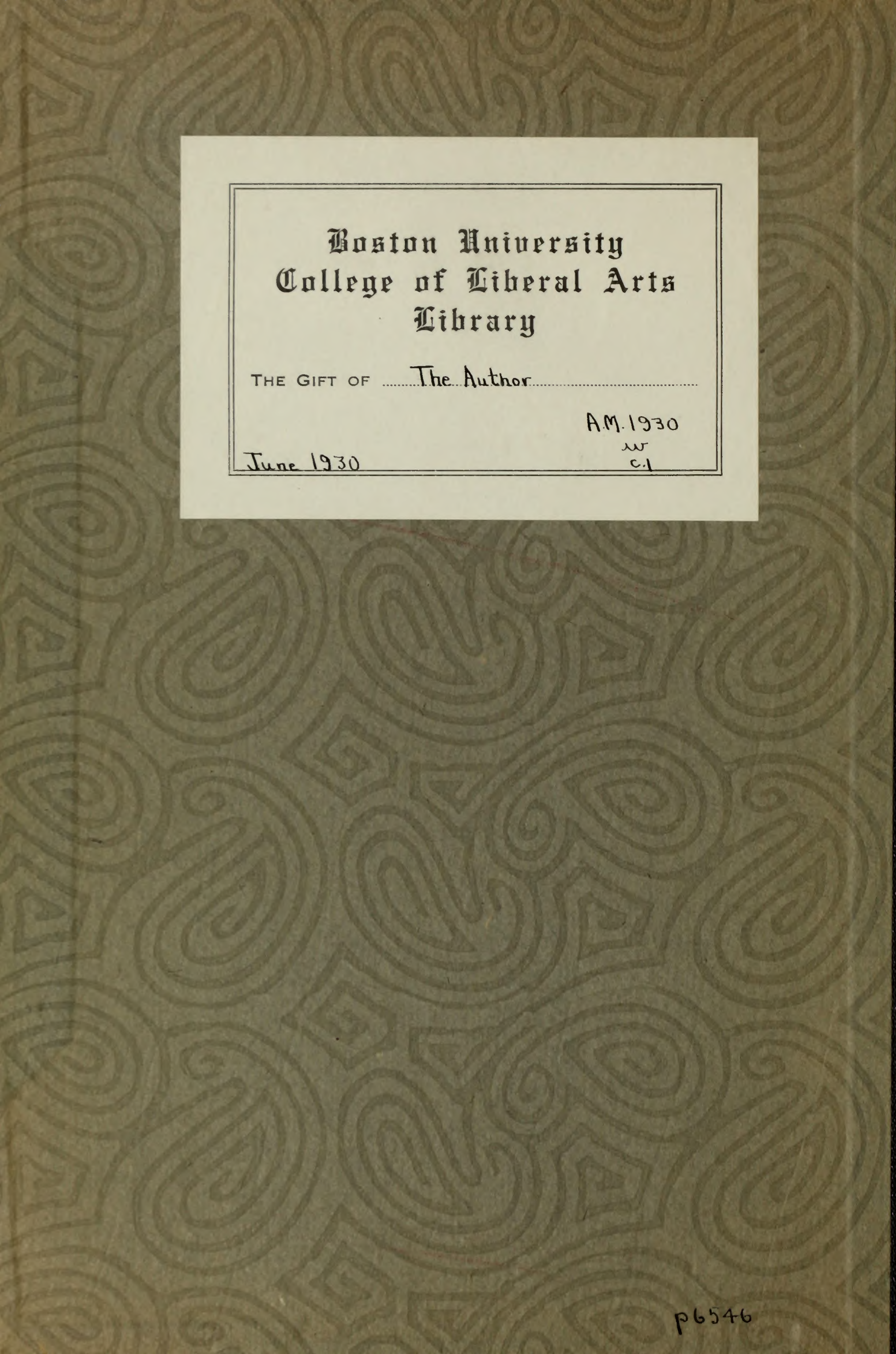

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Thesis

ESTRUS-PRODUCING SUBSTANCES:
THEIR OCCURRENCE IN ANIMAL AND VEGETABLE MATERIAL

Submitted by

Elisabeth Wellington Walker

(S.B., Boston, 1924)

In partial fulfilment of requirements for

the degree of Master of Arts

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I. Estrus in the mouse Page 1

A. Observations of vaginal spread

1. Guinea pig

2. Rat

3. Mouse

B. Estrus rhythm

1. Age of occurrence

2. Phases 2

a. Diestrus

b. Proestrus

c. Estrus

d. Metestrus

3. Duration

4. Cytological characteristics

5. Modification of estrus 3

a. Cold

b. Corpus Luteum

c. Chemicals

C. Effect of castration on estrus 4

D. Irregularities in the cycle

1. Vitamin A deficiency

2. Regeneration

II. Estrus-producing hormones 6

A. Discovery

Digitized by the Internet Archive
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https://archive.org/details/estrusproducings00walk

B. Sources Page 6
- 1. Ovary 7
- 2. Follicular fluid
- 3. Corpus Luteum
- 4. Placenta 9
- 5. Blood
- 6. Urine 10
- 7. Amniotic fluid 11
- 8. Plants 12
- 9. Eggs
- 10. Feces
- 11. Bile
- 12. Milk
- 13. Testes
- 14. Liver

C. Extraction 13
- 1. Lipoid solvents
- 2. Other methods 14

D. Purification

E. Properties 16
- 1. Solubilities
- 2. Stability 18
- 3. Chemical

F. Assay 20
- 1. Methods
- 2. Rat unit 21
- 3. Route of administration 22
- 4. Spacing of doses 23

5. Number of animals Page 24

6. Criteria of positive reaction

7. Individual variations 25

8. Technic for standardization 26

9. Sources of error 27

G. Physiological effects

1. Production of estrus 28

2. Mammary stimulation 29

3. Effect on isolated uterus 30

4. Abortion 31

5. Antimasculine effect

6. Effects on lower animals 32

7. Metabolism

8. Activity 33

9. .Blood chemistry

10. Tumors

11. Therapy 34

III. Methods 35

A. Experimental animals

1. Breeding

2. Care

3. Diet

4. Castration 36

B. Technic of tests

1. Preliminary smear 37

a. Methods

b. Readings 38

c. Errors

2. Injections Page 39
    a. Location
    b. Interval
    c. Test smears
IV. Results
  A. Blood 40
  B. Urine 42
  C. Tissues 45
  D. Chemicals 46
  E. Plants 47
V. Conclusions 50
VI. Summary 52
VII. Bibliography

Note: References to literature are cited by name of author and year of publication in the text, e.g., (Jones, 1926), following the convention used by the American Journal of Physiology. This has been done with the permission of the major instructor in order to avoid a cumbersome number of footnotes.

ESTRUS-PRODUCING SUBSTANCES: THEIR OCCURRENCE IN ANIMAL AND VEGETABLE MATERIAL

All attempts to study the sexual cycle of the female guinea pig had failed up to 1917 at which time it occurred to Stockard and Papanicolaou to observe the structure and changes in the uterus and vagina microscopically. This led to the discovery of regular cytological changes in the vagina - the estrus rhythm or cycle. Thus a method was evolved of diagnosing the stages of estrus in animals showing only slight external signs of their condition by a histological examination of the cell contents of the vaginal fluid. The cellular content of this fluid changes characteristically as the cycle progresses. This method has been applied to the correlation of estrual phenomena in the genital organs of the rat (Long and Evans 1922) and the mouse (Allen 1922). The estrual cycle of the mouse and rat is of 4 to 6 days' duration and this short period makes them especially suitable as test animals and furthermore this test does not involve sacrificing the animals.

The first estrus occurs with the opening of the vagina. In the case of the white mouse Engle and Rosaco (1927) found it to be from the 28th to the 49th day, the norm being the 35th. Kirkham (1920) mentions the fact that mice are sexually mature at six weeks. These statements are borne out by my own findings. The first ovulation occurs at this time and mating can take place.

The estrus cycle consists of four distinct phases: diestrus, a period of relative quiescence lasting from less than a day to as long as 14 days (this interval shows a greater variation than any other stage but is usually from 1 to 3 days); proestrus, a period of augmented growth and congestion, which may be less than a day, approximately 18 hours; estrus, a period of sexual excitement, lasting 1 or 2 days (sometimes 4); and metestrus, a period of return to the diestrus condition and lasting from 12 hours to 2 days. A complete cycle lasts on the average from 4 to 6 days (Parkes 1928). Allen (1922) found that the length of the cycle varies. In the brown mouse it is 6 days, while in the albino only 4. He suggests that "with the loss of the determiner for color a shorter cycle has been effected."

The cytological characteristics vary a great deal during the different stages. It is upon these variations that we base our tests for estrus-producing substances. During diestrus, the smear is made up of a few cornified epithelial cells, a few nucleated epithelial cells and an abundance of polymorphonuclear leucocytes. As this stage merges into proestrus, the number of leucocytes greatly diminishes, and we have a smear composed almost entirely of nucleated epithelial cells. Estrus is characterized by the presence of cornified cells only. During metestrus there is a decrease in cornified cells and a heavy infiltration of leucocytes in the vaginal content.

Stage	Duration	Content of vaginal smear
Diestrus	1-3 days	Few cornified epithelial cells Few nucleated epithelial cells Many polymorphonuclear leucocytes
Proestrus	18 hours	Nucleated epithelial cells
Estrus	42 hours	Cornified cells
Metestrus	12-48 hours	Few cornified cells Many leucocytes

Temperature influences the length of the cycle in rat and mouse. Lee (1926) observed that at 22° C. length of the cycle in rats was 4.8 days, while at -6° C. it might be as long as 18 days. Parkes and Brambell (1928) mention the fact that keeping the animals at 0° C. lengthens the period. Allen (1922) kept 4 mice at 40-55° F. and found that the duration of the cycle was not affected. The mice built an elaborate nest in which they spent most of their time.

Extracts of corpus luteum cause diestrus periods. Haterius and Pfiffner (1929) produced in rats diestrus lasting from 6 to 22 days by injection of extracts of corpus luteum. Parkes and Bellerby (1927) obtained similar results. Macht, Stickel and Seckinger (1928) inhibited the cycle in guinea pigs by daily injections.

Certain inorganic substances, such as thallium (Buschke, Zondek and Berman 1927), thallium and fluoride (del Castillo 1928) inhibit the cycle. 0.05 mgm. of sodium fluoride per day stops the cycle, which usually reappears 12 days after cessation of treatment. Estrus returns from 1 to 8 weeks after discontinuing thallium.

Removal of the ovaries causes cessation of the estrus cycle. Estrus or proestrus stages are not normally observed in completely castrated animals after the second day.

Under certain abnormal conditions cornified cells may appear in the vaginal smears of castrated mice. This is a source of annoyance and possible error in the assay of ovarian hormone preparations. There are two causes for this anomaly; a diet inadequate in vitamin A (Evans 1928, Coward 1929) and the regeneration of ovarian tissue. Cornification of the vaginal epithelium is characteristic of vitamin A deficiency in un-castrated animals as well. That the ovary is not involved is indicated by the continuation of the appearance of cornified cells throughout pregnancy. In vitamin A deficiency the cornification is not rhythmical but continuous (Evans). Coward reports that in some non-castrated cases there are persistent leucocytes rather than cornified cells.

The greatest difficulty is not that arising from vitamin A deficiency but is consequent to what we may call regeneration. The question whether or not regeneration follows complete ovariectomy is one which has attracted much attention recently. In 1925 Davenport reported 64% regeneration in the mouse following excision of one or both ovaries. In 1927 Parkes, Fielding and Brambell in 11 cases out of 121 found signs of spontaneous estrus and in 8 of these new ovarian tissue was seen under the microscope. They state that initial removal was complete enough to justify the view that regeneration took place from non-ovarian structures. In 1927 Hanson and Heys

found that regeneration occurred in 8 female rats out of 105. They assert that in 6 of the 8 the castration was successfully performed. Pallot in 1928 got 16.6% regeneration in the rat. He concluded that regeneration from peritoneal epithelium is probable rather than the existence of aberrant ovaries. In 1928 Haterius studied the regeneration of ovarian tissue in the mouse explaining it on the basis of incomplete removal. Marrian and Parkes (1928) got 2-3% regeneration after removing the top of each cornu in addition to the ovaries. Frank (1929) removed not only the ovaries, but also the adjacent peritoneum and Fallopian tube. He got regeneration in 1 out of 200. Recently a paper by Pencharz (1929) enlightens us considerably on the subject. He found that regeneration is no more likely to occur when the ovary alone is removed, capsules and oviduct being left intact. His work proved that "Ovarian tissue in the rat and mouse, providing all ovarian substance has been excised, does not arise 'de novo' from non-germinal tissue". The cases of regeneration obtained by other workers were probably due to unintentional incomplete ovariectomy. Pencharz states, "following partial ovariectomy the fragment left intact may undergo extensive hypertrophy and the hypertrophied fragment may become functional as indicated by the fact that pregnancy followed in 4 cases and that litters were born in 3 instances". Kahnt and Doisy (1929) were little troubled with positive smears and concluded that if the rats were carefully ovariectomized regeneration would not interfere with successful assay.

Discovery

In 1912 Iscovesco published two papers in which he stated that using organic solvents he had obtained a lipoid extract from the corpus luteum and the ovary. When these extracts were injected into rabbits they produced a hyperplasia and congestion of the uterus. In 1914 Okintschutz arrested uterine degeneration after double ovariectomy by injections of ovarian extracts. In 1915 Hermann and also Frank and Rosenbloom extracted a hormone from the placenta and corpus luteum which also produced marked growth in the uterus of the rabbit. Allen and Doisy (1923) injected follicular fluid from the sow into rats and mice, which resulted not only in stimulating growth of the uterus in the animals but also in increased proliferation in the vagina of a castrated animal. Thus these workers were able to apply the vaginal smear reaction (1922) in the mouse for testing their preparations without killing the animal. Upon injection of follicular fluid the changes in the vagina of castrated female rats and mice are similar to the changes which take place during normal estrual cycles in a non-castrated animal, namely proestrus, estrus, metestrus and diestrus. From then on many workers studied the sources of this estrus-producing substance.

Sources

The presence of an estrus-producing substance in whole ovary has been confirmed by every investigator who has worked on this problem since the time of Iscovesco (1912). The outstanding contributors have been the following:

Iscovesco (1912) ovary of the sow
Herrmann (1913) ovary of the cow
Allen, Whitsett, Hardy and Kneibert (1924)
ovary of the hen
Allen and Doisy human ovary
Loewe, Lange and Kaer (1929) ovary of the frog
Allen, Pratt, Newell and Bland (1930) human ovary

A similar agreement in results has appeared in regard to the content of follicular fluid in estrus-producing material.

Frank (1922) follicular fluid from cow
Allen and Doisy (1923) from sow
Seaborn and Champy (1923) from mare
Allen, Pratt and Doisy (1925) human
Parkes and Bellarby (1926)
Pratt (1927)
Kaufmann and Dunkel (1927) from cattle
Truffi (1927)
Doisy, Veler and Thayer (1929) human

In regard to corpus luteum, there is a wide discrepancy in the results of different investigators. A large group has reported the active material in corpus luteum.

Iscovesco (1912) corpus luteum of sow
Fellner (1912)
Herrmann (1913)
Frank and Rosenbloom (1915) of cow
Frankel and Fonda (1923)
Allen and Doisy (1925) human

Allen, Pratt and Doisy (1925) human

Frank and Gustavson (1925) hog

Zondek and Ascheim (1925)

Pratt and Allen (1926) human

Ascheim (1926) human

Pratt (1927)

Payne, Van Peenan and Cartland (1928) cow

Frank, Gustavson, McQueen and Goldberger (1929)

Allen, P ratt, Newell and Bland (1930)

A second group of workers obtained negative results from extracts of corpora lutea.

Allen and Doisy (1923)

Doisy, Ralls, Allen and Johnston (1924)

Allen et al. (1924)

Johnston and Gould (1926)

Allen and Doisy (1925) cow and sow

Pratt and Allen (1926) cow and pig (note that same authors found human corpora lutea positive)

Kaufmann and Dunkel (1927) cattle

Parkes and Bellarby (1927) cattle

A third group obtained extracts which were definitely estrus-inhibiting.

Papanicolaou (1926) guinea-pigs

Macht, Stickel and Seckinger (1928)

Haterius and Pfiffner (1929)

Very recently W.M. Allen (1930) has obtained an extract from corpora lutea which produces "progestational proliferation" but is differentiated from estrin in that it is destroyed by alkalies.

The weight of the evidence tends to the opinion that human corpora lutea contain an estrus-producing substance; the activity of the corpora lutea of other species is still in dispute. Pratt and Allen (1926) believe that the difference in hormone content of the corpora lutea of primates from those of lower mammals is a determining factor in some of the differences in the menstrual cycle.

Working chiefly with human material, many workers have demonstrated estrus-producing material in the placenta.

Fellner (1913)
Frank and Rosenbloom (1915)
Herrmann (1915)
Allen, Pratt and Doisy (1925)
Ascheim (1926)
Dickens, Dodds and Brinkworth (1927)
Brouha and Simonnet (1927)
Parkes and Bellarby (1927)
Dodds (1928)
Allen, Dickens, Dodds and Howitt (1928)
Phillipp (1929)

The presence of estrus-producing substance in circulating blood has been demonstrated by many workers.

In non-pregnant subjects (varying with the menstrual cycle)

Loewe (1925)

Frank, Frank, Gustavson and Weyerts (1925)
(sows in estrus)

Frank and Goldberger (1926 and 1928)

Fels (1926)

Hirsch (1928)

Wildebrush and McClendon (1929)

Janney (1929)

In pregnant subjects

Fels (1926)

Ascheim (1926)

Smith (1927)

Hirsch (1928)

Trivino (1928)

Janney (1929)

In male subjects

Dohrn (1927)

Frank and Goldberger (1928)

Hirsch (1928)

In cord blood

Loewe and Voss (1926)

Ascheim (1926)

Phillipp (1929)

The demonstration of the material in circulating blood led to its discovery in the urine.

Urine of non-pregnant subjects

Loewe and Lange (1926)

Laqueur (1927)

Wildebrush and McClendon (1929)

Of pregnant subjects

Veler and Doisy (1928)

Zondek (1928)

Doisy, Veler and Thayer (1929)

Veshnyakov and Lipschütz (1929)

Of male subjects

Laqueur, Dingemanse, Hart and de Jongh (1927)

Loewe, Voss, Lange and Wähner (1928)

de Jongh and Laqueur (1929)

Fee, Marrian and Parkes (1929)

Of the new born

Brühl (1929)

Phillipp (1929)

Of pregnant cows

Hisaw and Meyer (1929)

Nibler and Turner (1929)

The above may be considered as the chief sources. The most potent materials seem to be follicular fluid, placenta and the urine of pregnant subjects.

Various other materials have been demonstrated as containing estrus-producing substances in greater or less quantities.

Amniotic fluid

Brouha and Simonnet (1927)

Phillipp (1929)

Plant materials

Fellner (1921) yeast

Fellner (1925) rice and wheat

Dohrn, Faure, Poll and Blotevogel (1926)
potatoes and beets

Loewe, Lange, and Spohr (1927)
willow and water-lily

Glimm and Wadehn (1928) yeast

Frank (1929) tomato-juice

Eggs

Fellner (1925) birds' and fish

Jaffe and Ranssweiler (1926) lecithin

Frank (1929) yolk of hen's eggs

Feces

Dohrn and Faure (1928) feces

Gsell-Busse (1929) meconium

Bile

Gsell-Busse (1928) (1929)

Dingemanse and Laqueur (1929)

Milk

Brouha and Simonnet (1927)

Testis

Fellner (1925)

Laqueur, Dingemanse, Hart and de Jongh (1927)

(Frank reports negative results(1929)

Liver

Dingemanse and Laqueur (1929)

The extraction of the estrus- producing substances has been accomplished in the majority of studies by the use of organic fat solvents. Iscovesco (1912) used alcohol to prepare his first extract, and purified by using various solvents; ether, chloroform, acetone, etc. Hermann (1913, 1915) followed a similar scheme, as did Morley (1917), Allen and Doisy (1923), Dosiy et al.(1924). Although Zondek and Brahn (1925) and Zondek (1928) used quite different methods of preparation, the initial extraction was with a fat solvent. A rather unusual solvent, butyl alcohol, was introduced by Allen, Dickens, Dodds and Howitt (1928). Nibler and Turner (1929), using cows' urine, reduced the extraction process to an extreme of simplicity by merely agitating the urine with an oil and injecting the oil. (An interesting finding of these investigators is that the hormone content of the urine correlated rather well with milk and butter fat production.) This method was applied to human urine by Doisy, Veler and Thayer (1929). Benzol as a solvent was introduced by de Jongh and Laqueur (1929).

Three workers have used methods other than the use of fat solvent for primary extraction. Biedl (1927) separated the hormone from a suspension of ovarian material by electro-dialysis, the hormone going to the cathode. Dickens, Dodds and Brinkworth (1927) extracted active material from placenta by treatment with hot 7% HCl, adjustment of pH to 3, passage through a Sharples centrifuge, and successive treatment with saturated picric acid, acid alcohol and acetone. Wiles (1929) found that the hormone could be quantitatively adsorbed from urine by

calcium phosphate. Walker (unpublished data) has also shown that aluminum hydroxide, barium sulfate, nickel hydroxide and nickel sulfide are capable of adsorbing the estrus material from pregnant urines.

Recently Goldhammer (1929) has described a method of isolation of estrus-producing material from placenta or urine by precipitation with antimony pentachloride. This is not an adsorption, but appears to be dependent uponthe formation of a definite compound. This reaction is characteristic of the sterols in general and points to a chemical identification of estrin as allied to the sterols.

Purification of the estrus-producing material has largely been accomplished by the use of various solvents, depending upon differences in solubility. The work of Iscovesco (1912) is typical in this respect, and the work of later investigators has been modelled to a great extent upon his methods of fractionation. By such use of solvents Doisy, Ralls, Allen and Johnston (1924) were able to free their product entirely from cholesterol. Zondek and Brahn (1925) utilized the solubility of the active material in dilute acetic acid in the purification of their crude extracts. The extraction and purification by dilute hydrochloroc acid used by Dickens, Dodds and Brinkworth (1927) was mentioned in the last paragraph. Zondek (1928) produced the purest product obtained up to that time by suspending the crude extract in water and extracting with ether. Repetition of this process, saving the ether extracts, produced a fraction of which 0.001 mgm. contained a mouse unit.

A later modification by Zondek, based on the fact that the hormone could be extracted by ether from an alkaline solution (1928) led to the introduction of the step of saponification of the crude extract with strong NaOH, after which it was suspended in water and extracted with ether as before. By this means a clear, colorless and odorless aqueous solution was prepared, with high activity. From such active concentrates, Marrian (1929) was able to crystallize a solid saturated alcohol, M.P. $232-235^{\circ}$ C., $C_{19}H_{32}O_2$ or $C_{20}H_{34}O_2$, which, however, was inactive. The mother liquors from this cryatallization were highly active. The fact that the active material could be extracted from an alkaline medium with ether was considered to be evidence of the unsaponifiability of the substance. Funk (1929) however, reported the preparation of sodium, potassium, ammonium and barium salts, which were active and soluble in ether. Wildebrush and McClendon (1929) altered this procedure by making the original extract directly from alkaline material (blood or urine), obtaining a first extract of considerable purity. Doisy, Veler and Thayer (1929) reported the isolation of crystalline material of high activity, by crystallization from butyl alcohol. The details of the preparation and the chemical characteristics of the product were not included in the brief published report. Butenandt (1930) by distillation in vacuum and recrystallization, obtained a crystalline product with a potency of eight million mouse units per gram. The melting point of this compound and its empirical formula correspond closely to those of the solid alcohol

isolated by Marrian (1929) and found to be inactive.

There is considerable confusion in regard to the chemical properties of the estrus-producing hormone, a result mainly of the fact that it has never yet been definitely isolated (not considering the recent work of Butenandt, which still lacks verification). There is, however a certain amount of agreement in some points, and I will endeavor to summarize the present state of our knowledge in this respect, pointing out the details in which there is disagreement.

All workers agree that the active material is soluble in the usual organic fat solvents. This observation dates back to Iscovesco (1912) and has been confirmed by all workers since. Giesy (doctor's thesis, 1920, quoted by Frank 1929) sums up the consensus of the solubility experiments: "The substance under investigation was found to be soluble under definitely prescribed conditions, in benzene, chloroform, carbon tetrachloride, absolute and 95% alcohol, ether, ethyl acetate, carbon bisulfide, pyridine, acetone, acetyl chloride, and hot acetic anhydride. It was somewhat soluble in 50% alcohol and glacial acetic acid. Under certain conditions it was soluble in water and benzin (petroleum ether)".

The low solubility in petroleum ether has been used as a basis of separation from cholesterol, which is very soluble in petroleum ether (Doisy, Ralls and Jordan, 1926) (Ralls, Jordan and Doisy, 1926).

The solubility of the active material in water is a question which has resulted in considerable controversy.

Doisy, Ralls, Allen and Johnston (1924) state definitely that it is insoluble in water. Zondek and Brahn (1925) obtained an active water soluble preparation after vigorous saponification of the fats of the crude extracts. Since then, most workers have prepared, or at least admitted the existence of, water-soluble forms of the estrus-producing hormone. None of these preparations were claimed to be pure. The crystals isolated by Butenandt (1930) are soluble in organic solvents, but not in water, resembling in this respect the solid alcohol isolated by Marrian (1929) and found inactive.

The quantitative aspects of the solubility question have not been investigated. The material seems to be almost infinitely soluble in the organic solvents. Water preparations as concentrated as 100 mouse units per cc. are easily prepared (Walker, unpublished observations).

It should be emphasized that there exists a preferential solubility as regards the organic solvents. The hormone can be completely removed from aqueous solutions by extraction with ether (Frank, 1929).

Glimm and Wadehn (1929) believe that the estrus-producing substance may exist in two forms, one ether-soluble and one ether-insoluble, with the former predominating. Continued boiling with alkali, say these authors, converts the soluble form into the insoluble. This reaction can be reversed by treatment with acid.

Funk (1929) believes that the water-soluble hormone is a salt which is also soluble in ether.

Stability.

The extreme thermostability of the estrus-producing material is remarkable, in comparison with most hormones. Laqueur found that it resisted a temperature of 360° C. when in oil solution, and of 170° C. when in aqueous solution (1927).

Light has been reported to have a deleterious effect (Doisy and Jordan, 1926) although in my own experience, untra-violet radiation for half-hour periods had no effect.

Tryptic digestion has no effect on the hormone (Doisy, Ralls, Allen and Johnston, 1924), nor does peptic digestion (Laqueur, 1927).

The hormone is not affected by 20% sulfuric acid or sodium hydroxide (Laqueur, 1927) in the absence of oxidizing agents. The activity is quickly destroyed by oxidation. In this respect it is necessary to see that the ether used in extractions is free from peroxide (Thayer, Jordan and Doisy, 1928).

Aqueous solutions deteriorate on standing, even when protected from light and air. Frank (1929) reports that they are stable only for a few days. My experience has been that they are good for several weeks if kept at room temperature. They lose activity more rapidly if kept in a refrigerator.

Chemical Composition.

The crystals isolated by Butenandt were analysed by a micro-combustion with the result: C 78.13%, H 8.13%; melting point 240° C. All earlier analyses were made on

uncrystallized material and are of very doubtful value. Fränkel and Fonda (1923) reported an empirical formula $C_{32}H_{52}O_2$. Ralls, Jordan and Doisy (1926) found 0.9% nitrogen and based a molecular weight of 1500 on the percentage of nitrogen. By depression of the freezing point they found a molecular weight of 475. Simonnet (1927) prepared a nitrogen-free product of high concentration.

Reactions.

The most important chemical reaction is the susceptibility to oxidation already mentioned. Chemical methods of detection or identification have not been reported. Doisy, Veler and Thayer (1929) mention color reactions, but do not give details. Butenandt (1929) has tried various reagents (e.g. acetic anhydride and dulfuric acid) in the hope of finding a color reaction for his crystals, but has so far been unsuccessful. Goldhammer (1929) develops a precipitate and color with antimony trichloride, but this test is not specific.

The hormone is believed to be unsaturated by Hartmann (1926) on account of its ability to absorb bromine. Frank (1929) confirms the fact of bromine absorption, with the formation of a compound toxic to mice. If iodine is used, there is no effect on either toxicity or hormone activity. Frank believes that the hormone has the structure of a gamma hydroxy aldehyde in the lactone form. He bases his assumption of an aldehyde group on the fact that both oxidation and reduction (catalytic hydrogenation) destroy the activity, and on the apparent formation of a bisulfite addition product. The oxidized product can be reactivated

by mild catalytic hydrogenation: simple acids cannot be reduced to aldehydes in this manner, but "if the hypothetical aldehyde contained an hydroxyl group in the gamma position, the acid formed on oxidation would form a gamma lactone and these lactones are easily reduced back to aldehydes".

To the consideration of the chemical properties should be added: 1. The ease with which the active material is adsorbed from aqueous solution, indicating that it is possibly in the colloidal state. Even passage through filter paper (laqueur, Hart and de Jongh, 1926) will remove some of the hormone. 2. In contrast, the hormone appears to be diffusible through a parchment membrane (Trivino, 1928). 3. The hormone appears to be subject to electrocataphoresis, passing to the cathode (Biedl, 1927).

There are four methods of assay which have been used for the female sex hormone. They are:

1. Uterine growth
2. Uterine contractility
3. The chromophil reaction
4. The vaginal smear

The recent work of Collip (1930) has indicated that the hormone which stimulates uterine growth is not identical with estrin. Hence a certain uncertainty is attached to the methods involving uterine growth. Such methods were used in the earlier studies of female sex hormone by Frank and Rosenbloom (1915). To a less extent, Collip's work has invalidated the uterine contraction tests of Frank, Bonham and Gustavson (1925). This test, involving the sacrifice of the animal, delicate apparatus and measurements, has never met with wide use among other investigators.

Blotevogel, Dohrn and Poll (1926) observed that the number of chromophil cells in the para-cervical ganglion of Frankenhäuser depends on the stage of the estrus cycle. The ratio of chromaphil to total ganglion cells in the resting stage is 0.025. It increases with estrus and is at its maximum (0.15) at parturition. It is reduced to less than 0.01 by castration. Injection of the female sex hormone causes an increase in the number of chromophil cells, approaching the level of pregnancy. Dohrn, Faure, Poll and Blotevogel (1926) used this method in assaying the "tokokinin" (estrin) content of various plant materials (sugar beets, potatoes, cherries yeast).

The method of choice, since the time of Allen and Doisy (1923) has been the observation of estrus in the castrated rat or mouse, using the vaginal smear as a criterion of estrus. This method has undergone many modifications and elaborations in the hands of later workers. The basic concept of a unit based on the amount of material necessary to produce estrus in a standard animal was proposed by Allen and Doisy (1923). A rat unit (R.U.) or mouse unit (M.U.) is the amount necessary to produce the estrus changes in a castrated animal.

Allen and Doisy (1923) have defined the rat unit as the amount of substance necessary to produce a full change from the negative to the estrual vaginal smear in a castrated rat of approximately 140 grams (±20 grams) in weight. Bugbee and Simond (1928) define the potency of preparations in terms of rat units per cc. on a basis of minimum effective dose (in cc.) and weight of the rat.

Let Q equal the minimum number of cc. which produces estrus.

Let W equal the weight of the rat in grams.

Then R.U. per cc. equals $\frac{1}{Q \times \frac{140}{W}}$ equals $\frac{W}{140\ Q}$

The mouse unit has not been so strictly defined in terms of body weight, but is used to indicate the amount of material necessary to produce estrus in a castrated mouse. The restrictions placed on these definitions by different workers will be discussed in succeeding paragraphs.

The necessary conditions for accurate assay of estrus-producing material have been the subject of many investigations. These will be discussed under the topics: 1. Route of administration. 2. Spacing of doses. 3. Neceessary number of animals. 4. Age of animals. 5. Weight of animals. 6. Criteria of positive reactions. 7. Individual variations and sources of error.

The route of administration is predominantly subcutaneous. The small size of the animals rules out the intravenous route. Evans and Burr (1926) observed that there was less uniformity of results with intraperitoneal than with subcutaneous injections, Kahnt and Doisy (1928) confirming this finding. Marrian and Parkes (1929) in their critical study of the assay of estrin, found no advantages in the intraperitoneal route, and recommend the subcutaneous. Oral administrations have proven effective, but less efficient than subcutaneous. Neither peptic nor tryptic digestion destroys the activity (Doisy, Ralls, Allen and Johnston, 1924) (Laqueur, 1927).

Loewe (1925) found that his preparations were effective by mouth, but required a twenty-fold increase in dose. The preparations obtained from commercial houses were ineffective by mouth. This same ratio between oral and subcutaneous doses was corroborated by Loewe, Lange and Faure (1926). Laqueur, Hart and de Jongh (1927) found a hundred-fold increase in dose necessary when administered to rats by mouth, but only eleven-fold with mice. The latter finding was checked by Laqueur and de Jongh (1928). Hannan (1928) found a sixty-fold increase necessary. Banti (1929) using rats found an eleven-fold increase necessary when given by mouth.

On account of the rapid destruction or elimination of the estrus-producing material, repeated doses have been found more effective than a large single dose. Zondek (1926) spreads the injections over 48 hours, giving six injections. Bugbee and Simond (1926) recommended three injections at four hour intervals, but later (1928) gave eight injections in a 48 hour period. Laqueur (1927) used the same intervals as Zondek. Kahnt and Doisy found the intervals first used by Bugbee and Simond (three injections four hours apart) satisfactory (1928). Marrian and Parkes (1929) state that "splitting the dose into a series of injections greatly increases the percentage response. ...The optimum time over which such injections should be spread is 24 to 36 hours....The method finally adopted for routine testing is to give four injections at 12-hour intervals, i.e. to cover 36 hours".

The number of mice (or rats) necessary for an accurate assay is given as twelve by Laqueur (1928) and

by Bugbee and Simond (1928). Allen, Dickens, Dodds and Howitt (1928) believe twenty mice are necessary. It is of course quite permissable to use a smaller number in preliminary determinations.

In regard to age, for mice Bugbee and Simond find best results with mice aged from three to eleven months.

Although the rat unit has been based on the weight of the rats, the investigators who have used mice find no correlation between weight of animals and size of effective dose. In regard to the effect of weight, in rats, see the discussion above of the Bugbee-Simond rat unit. Laqueur and de Jongh (1928) and Marrian and P arkes (1929) both report that in mice the body weight can be neglected. In my experiments I attempted for a time to correlate dosage with weight of mice, but found no satisfactory agreement.

As to whether or not a given reaction is positive or only doubtful depends to a large extent upon the judgment of the observer..Because of this and of the latitude of criteria used by the various investigators many discrepancies arise. Frank and Goldberger (1928) base their readings on a scale running from 0 to 4.

0 --- all leucocytes

1 --- few nucleated epithelial cells
many leucocytes

2 --- many nucleated cells, some leucocytes

3 --- many nucleated epithelial cells
occasional leucocytes

3 --- no leucocytes
large number of nucleated epithelial cells

4 --- all dead epithelial cells

The results of Laqueur and de Jongh (1928) depend upon the disappearance of leucocytes in the vaginal smears stained by the Giemsa method.

Over 95% cornified cells --- double positive

From 95% to 50% --- single positive

Since the complete absence of leucocytes is rare, Laqueur allows 5% in a positive reactions. A smear containing more than 5% is either doubtful or negative - doubtful if two thirds the epithelial cells have nuclei, otherwise negative.

Kahnt and Doisy (1928) have a similar scale. They assume that a predominance of cornified cells with some nucleated epithelial cells and a very few leucocytes should be taken as a positive reaction.

On the other hand, Marrian and Parkes (1929) insist upon a smear "consisting of all cornified cells or a mixture of nucleated and cornified cells. The complete absence of leucocytes and the presence of cornified cells have been rigidly enforced".

Most workers have observed the individual variation among animals in response to given doses of estrin. (Marrian and Parkes, 1929)(Laqueur and de Jongh, 1928) Variations from fully positive to fully negative reactions may occur with the same dose. Even the same animals on repetition of the dose may show inconsistent results.

Kostich and Telebakovitch (1929) found that the smears in spayed white mice show cyclic changes similar to normal estrus, but less intense. Brambell and Parkes (1927) also suggest that a low threshold for the hormone may occur periodically.

Coward and Burn (1927) in a group of ninety rats and seventy mice found the amount of variations among individuals to be as great as 1000%. In order to eliminate this individual element a fairly large number of animals (see page 24) should be injected. Kahnt and Doisy (1928) suggest the following technic for standardization:

1. Make daily smears for a two or three weeks' period before ovariectomy. Only those animals with normal cycles should be used.

2. Make daily smears for two weeks after operation but before the beginning of the injections. Discard any animal showing estrus or proestrus smears later than the second day after operation.

3. Prime the animals with two rat units.

4. One week later test the reaction of each animal by the injection of 1.3 rat units. If the smears are negative, discard the animal.

5. A week later test the reaction to 0.70 rat units; if positive smears occur, discard the animal.

6. Use each animal for no longer than four months.

7. In attempting accurate assays, prime all animals by injecting 1.5 rat units, if the reaction of the preceding week was negative.

8. Use a sufficient number of animals. If 75% of the animals injected with the same volume give a positive reaction, consider that the amount injected contained one rat unit.

This procedure is cumbersome for use in the average laboratory. I have obtained a remarkable uniformity of results by simply using a sufficient number of mice for each standardization.

From time to time a number of cornified cells may occur in an otherwise negative smear from a castrated mouse. Loewe and Lange (1926) maintain that in a full resting state a smear containing 30% cornified cells should never be exceeded. This fact was explained by Ascheim and Zondek in the same year, when they pointed out that the large number of cornified cells found by Loewe and Lange were probably obtained from their own fingers in making the swab with which to take the smear. Ascheim and Zondek show that the smear should be made with a platinum loop deep within the vagina in order to avoid as much as possible contact with the vestibular part of the vagina which always contains cornified cells.

In discussing the physiological effects of the estrus-producing hormone, a certain difficulty arises in differentiating the effects of estrin from the effects of other materials derived from similar sources. The recent work of Collip (1930) for instance, shows that the placenta contains a stimulant to uterine growth which is different from estrin, and can be completely freed from estrus-producing material.

In this discussion the attempt will be made to deal only with the estrus-producing hormone. In some cases there will be doubt as to whether the described effect is produced by estrin, or by some accompanying substance.

The most important effect is, naturally, the

production of estrus. To Allen and Doisy (1923) we owe the development of the vaginal smear method of the detection of estrus. At the same time they established the presence of estrin in the follicular fluid of the ovary of the sow. This is the first published article in which a definite estrogenic substance was tested, using a wholly objective test for estrus. The previous preparations of Iscovesco (1913), Fellner (1912), Herrmann (1913), Frank and Rosenbloom (1915), Fellner (1921) and Frank (1922) were tested on the basis of growth of the uterus, breasts or vulva of test animals (the rabbit in most cases). There is little doubt that these extracts were estrogenic; Fellner noted morphological changes in the uterus (of rabbits and guinea pigs) characteristic of estrus; the preparation of the extracts was carried out with fat solvents in essentially the same manner as we now proceed to prepare estrin. At that time there was no attempt to separate the action of the estrus-producing and the uterine growth-stimulating substances. Their complete separation was accomplished by Collip (1930).

Estrus produced by the action of estrogenic substances has been studied almost entirely by means of the vaginal smear. An exception has been the chromophil reaction of Blotevogel, Dohrn and Poll (1926) already discussed under assay. The phenomenon of estrus induced in castrated mice by injection of estrin has also been covered under that topic. In immature female rats (either castrated or not) a rapid maturation of the uterus and vagina takes place,

(Allen and Doisy, 1924) (Golding and Ramirez, 1928). This effect was brought about by Allen and Doisy by the injection of liquor folliculi (sow) and by Golding and Ramirez by the use of commercial ovarian preparations. The same effects on immature females were noted by Courrier (1924) with guinea pigs and by Truffi (1927) with rabbits and dogs, following the injection of liquor folliculi (mare). In senile mice, estrus was induced by Laqueur, Hart and de Jongh (1926) by means of their preparation of the estrus hormone, menformone. Lactating mice can be brought into estrus by injection of estrin (Parkes and Bellarby, 1927). An interesting experiment was performed by Courrier (1924) on the hedgehog, which has a long anestrus period during hibernation. Vaginal structural modifications characteristic of estrus were produced by injection of follicular fluid. In ovariectomized monkeys, following injection of ovarian extracts, reddening and swelling of the sexual skin and changes in the vaginal smears were noted by Robertson (1929).

What corresponds to an estrogenic action has been observed in birds by Loewe, Voss and Paas (1927). They found that injection of ovarian extract into old hens (two of them) was followed by a short period of active egg-laying.

Hartman, Dupre and Allen (1926) observed that follicular or placental extracts, estrogenically active, stimulated the growth of the mammary glands. Following ovariectomy, the usually observed atrophy of the glands could be prevented by injection of estrus-producing sub-

stance. Laqueur, de Jongh and Tausk (1927) were able to duplicate these results with their "menformone", using immature rats (male or female) or mature male rats. The secretory parts of the mammae were the parts affected. In 1928, Laqueur, Borchardt, Dingemanse and de Jongh postulated that menformone was the hormone which controlled the normal development of the mammary gland. Two more detailed reports by Brochardt, Dingemanse, de Jongh and Laqueur (1928) gave much evidence in favor of this theory. It was found possible by continued injections of menformone to produce abundant milk production from a male guinea pig. These same authors report increase in the size of the adrenals. Haterius (1928) was unable to produce quite such marked responses by the injection of placental extracts into male guinea pigs. He did, however, secure histological evidence of secretion and colostrum formation.

Estrogenic preparations have an effect on the isolated uterus. The experiments of Frank, Bonham and Gustavson (1925) and the method of assay based on them, have already been discussed under assay. Brouha and Simonnet (1926)(1927) observed that the subcutaneous injection of follicular extract in guinea pigs alters the response of the uterus to pituitrin (in vitro). The initial contraction becomes more rapid and is followed by a series of rhythmic contractions instead of an irregular clonus. In a second paper they report on the effect of liquor folliculi on the resting uterus of the rat in vitro. Additions of liquor folliculi (or extracts) augments the amplitude of contractions. With the guinea pig uterus there is an increase

in tonus without causing contractions. These same authors were able to induce estrus and rhythmic contractions of isolated uterine muscle by the use of washings from the uterus of the rat during normal estrus.

Abortion can be produced by injections of estrogenic material. M.J. Smith (1926) was able to interrupt pregnancy in the first five days (in the rat) by injection of extract of liquor folliculi. Larger doses were required as the pregnancy progressed. Parkes and Bellarby (1926) were able to terminate pregnancy in any stage, provided a sufficiently large dose was given. Pighini and Colonna (1929) injected liquor folliculi into a cow (1 cc. one day, 2 cc. the next) and observed that she expelled a dead fetus on the third day. It is difficult to reconcile these experiments with the well-substantiated fact that there is a large increase in the amount of circulating estrin in the blood as pregnancy progresses.

An antimasculine effect has been noted by a few observers. By injections of liquor folliculi, Truffi (1927) was able to produce atrophy of the male genitalia in rabbits and dogs. Borchardt, Dingemanse, de Jongh and Laqueur (1928) using "menformone" were able to check the development of male sex organs and characteristics and with high doses (20-60 M.U. daily) to produce a decrease in the sexual organs of adult males (rats, rabbits, guinea pigs). Engle (1929) injected urine from pregnant women into immature male rats, and observed marked hypertrophy of the

sex apparatus, except the testis. Injections of male urine produced a similar, but slight effect.

A few experiments have been made concerning the effects of ovarian hormone on the growth and behaviour of lower animals. The sugar metabolism of paramecia is increased by the presence of ovarian hormone (Estes and Burge, 1928). The permeability of the membrane of the egg of Fundulus is altered by follicular extract (Carroll, 1930). There is no evidence of any stimulation of cell proliferation in the embryo. Bisexuality and sex reversal in Amblystoma was not influenced by the presence of ovarian hormones (Humphrey, 1929). Ovarian tissue, and to a less degree, flesh from broody hens produced earlier development of frog tadpoles tan controls from normal hens (Moschini, 1929). The significance of these experiments is rather doubtful in our present state of knowledge.

Ovarian extracts have been repotted to have an effect on the basal metabolic rate. Zondek and Bernhardt (1925) based a standardization procedure on the increase in basal metabolism produced in castrated women. This observation app ears to be better suited to consideration as a physiological observation rather than as a practical method of standardization, hence was omitted in the discussion of assay. This finding is in disagreement with the work of Heyn (1927) who found no correlation of the basal metabolic rate with the menstrual cycle in healthy or dysmenorrhoeic women. He also observed that the decrease in basal metabolic rate after castration was temporary. That the hormone content of the blood varies with the menstrual cycle

has been demonstrated by Frank (1929), Janney (1929) and others. McClendon, Conklin, Wildebush and Wiles (1929) agreed with Zondek and Bernhardt in regard to the effect of injections of ovarian hormone on the basal metabolism. They also found a variation in B.M.R. with the menstrual cycle, following the same curve as the excretion of the hormone in the urine. Koehler (1929) partially contradicts these results. She was unable to produce any change in B.M.R. in eight cases of disturbed ovarian function. She concludes that the effects of castration, e.g., weight changes, are not the direct result of lack of ovarian hormones.

Voluntary activity is increased in ovariectomized rats during the periods of induced estrus (Bugbee and Simond, 1926) (Slonaker, 1927). Ovarian extract by mouth had no effect on activity (Durrant, 1925).

The effects of estrogenic material on the chemistry of the blood have been investigated. Ovarian hormone or liquor folliculi causes a rise in blood sugar (Kylin, 1926) (Rathery, Kourilsky and Gilbert, 1928). Blood calcium is lowered (Mirvish and Bosman, 1927) (Reiss and Marz, 1928). Blood cholesterol is increased proportionally to the amount of hormone injected (Reiss, 1928)(Mori and Reiss, 1928). Folliculin injected intravenously causes a rise in the alkali reserve without alteration of the pH (Imparato, 1929).

Some authors have attempted to trace the connection between ovarian hormones and tumor growth. L. Loeb (1919) believes that a hormone given off by the ovary regulates those tissue changes which lead to the development of cancer of the breast in mice. Murray (1927)(1928) was able to

cause male mice to develop spontaneous tumors by subcutaneous transplants of ovarian tissue. He definitely links the incidence of certain mouse neoplasms with ovarian secretion. On the other hand Fellner (1922) was able to protect mice against cancer and inoculated tumors with his "sexuallipoid" (from corpus luteum, placenta or testicle).

The use of estrogenic materials (usually ovarian in source) in medicine is hardly a part of the subject matter of this thesis. A few references will be given to show the general method of application and some of the results obtained by clinical investigators. No claims are made as to the completeness of the bibliography in this section. Ovarian hormone, derived from follicular fluid or placenta, has been successfully used in cases of amenorrhoea by Watrin (1925), Parhon (1927) and McClendon, Burr and Wildebush (1929). A placental extract caused improvement in a series of twenty-five menorrhagic patients (Jacoby, 1923). Disturbances at the menopause have been relieved by folliculin (Brouha and Simonnet, 1926) and by "amniotin", a preparation from the amniotic liquor of cows (Sevringhaus and Evans, 1929). Ovarian dysfunction and associated symptoms have yielded to treatment with ovarian hormone (Van de Walle, 1927) (Firestone, 1928). Cohn (1928) combined stimulation of the ovaries by diathermy and intravenous injection of ovarian extract in the treatment of the psychic symptoms of ovarian dysfunction.

In addition to the usual methods such as oral, subcutaneous and intravenous administration, Powers, Varley and

Morrell (1929) suggest the administration of the follicular hormone in a vaginal pessary, while Pratt and Schmeltzer (1929) have experimented with nasal spray.

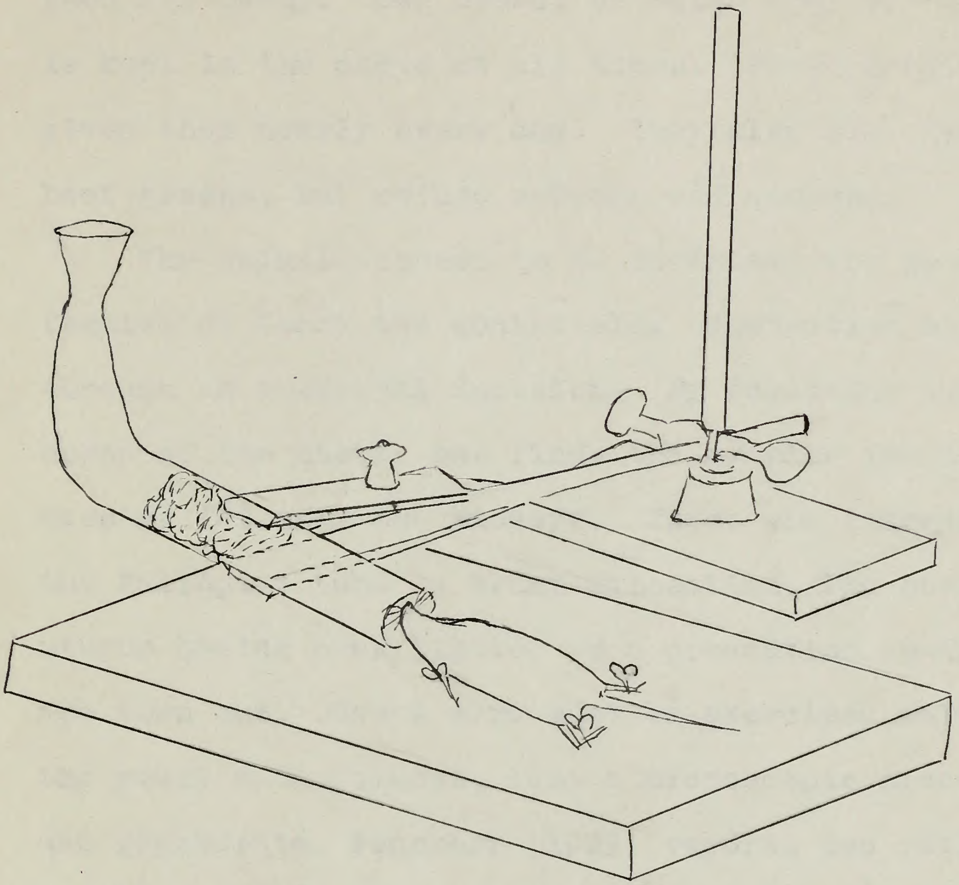
EXPERIMENTAL METHODS

The animals used in testing out preparations are albino female mice. The stock is bred for this purpose, I have found, in agreement with Engle and Rosaco (1927) that the vagina opens from the 26th to the 49th day. The mice usually breed almost as soon as the opening of the vaginal orifice appears. The period of gestation is from 18 to 21 days as reported by Kirkham (1920). Most of the litters are born on the 21st day; by the time the mice are two months old nearly all have had litters. The size of the litters varies from two to thirteen. The average number p er litter which reach sexual maturity is 5.5 . Unfortunately the sexes are not equally divided. From fifty litters approximately 56% were males. Therefore, in order to get at least ten female mice it is necessary to breed five mothers.

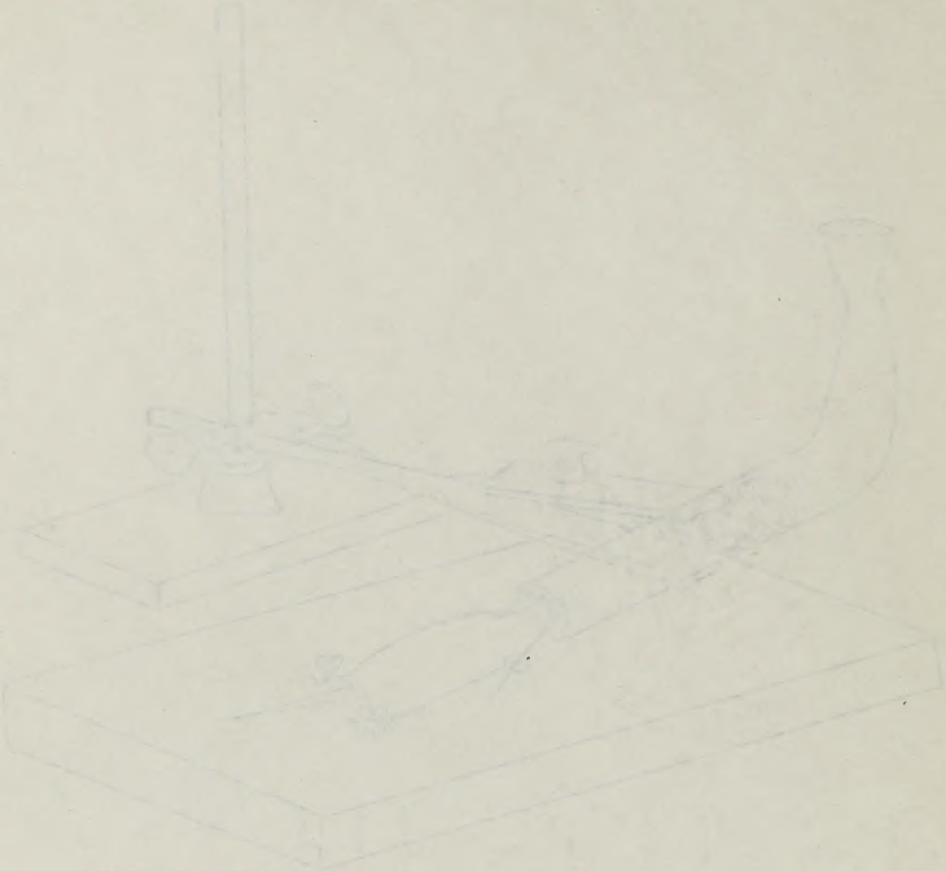
The mice are housed in wire cages, ten to a cage. These cages are placed in baking pans which have paper in the bottoms to facilitate cleaning. The mice are given paper ribbon (such as is used for packing) in which to nest. The cages are cleaned daily where there are a large number in a cage, less often in other cases.

The diet of the test animals is carefully watched. This consists of hominy mixture (salt, meat scraps, powdered milk, rolled oats and hominy) twice a week. Cod liver oil is mixed with this to avoid deficiency in

ETHERIZING APPARATUS



Apparatus made by W.C. Boyd
after specifications of
James C. Janney, M.D.



vitamin A which will cause a persistent cornification of the vaginal smear (Evans, 1928)(Coward, 1929). Twice a week they are given cracked corn and cheese, once bread and milk and once a seed mixture (sunflower seed, hemp seed and oats). Dog bread, of which they are very fond, is kept in the cages at all times. Fresh lettuce is given them nearly every day. They also like spinach and beet greens, but refuse cabbage and carrots.

The animals chosen to be castrated are healthy adult females at least two months old. Castration is accomplished through an abdominal incision. By following along the horns of the uterus one finds the ovaries imbedded in a mass of fat near the kidneys. These are removed as well as the Fallopian tube by blunt dissection. The horns of the uterus having been ligated as a precaution against hemorrhage are then cut. Great care must be exercised not to injure the ovary with forceps, lest a microscopic piece be broken and regenerate. Pencharz (1929) reports two rats as having had litters as a result of the regeneration of a bit of ovarian tissue left in situ.

As mice are extremely resistant to infection the operations are not absolutely aseptic, but for the most part care is taken to have everything sterile. The operation is carried on under ether anesthesia. A glass tube about one inch in diameter, bent nearly at right angles, is placed in a ring stand. The mouse's head is put into the horizontal part and ether is poured onto cotton in the vertical piece. In this way the anesthetic can be given by the

operator. This has proven very satisfactory and there are fewer deaths from anesthesia than when a second person administers the ether; the mice recover very quickly after the operation, in about two hours moving about and gnawing at dog-biscuits. The mortality from the operation is very low. The majority of the deaths which occur result from hemorrhage following the cutting of the toes. This is done to identify the mice. It is an unsatisfactory method on account of occasionally causing a death. In the future, mice will be marked with an ear punch. Mice having ascitic fluid or fluid in the uterus are poor operative risks.

If the mice operated are less than sixteen days pregnant the embryos die and are absorbed; if pregnancy is further advanced, abortion may occur or the mice may go on to term. (Harris, 1927)

A week after ovariectomy the mice are smeared daily for a week and if the spreads are consitently negative the mice are ready for use. Double ovariectomy one day before estrus does not prevent it (Brambell and Parkes, 1927). Hence the appearance of cornified cells in the smear two days after ovariectomy may be disregarded.

The vaginal smears are made with glass pipettes, one to a mouse. A few drops of luke-warm water are taken up in the pipette. In making the smear, one holds the mouse by the tail with the left hand, the mouse clinging to the cage with the front feet. With the right hand, one inserts the pipette into the vagina and aspirates the water back and

forth a few times. The contents of the pipettes is spread upon a glass slide, dried with heat, stained with 1% aqueous solution of thionin and examined under the low power of the microscope. Our scale of readings is as follows:

NEGATIVE		
	0	Almost 100% leucocytes
	0-1	All leucocytes except a few scattered nucleated epithelial cells
	1-2	20-50% nucleated epithelial cells
DOUBTFUL		
	2	50-75% nucleated epithelial cells
	3	75-100% nucleated epithelial cells
POSITIVE		
	3+	Nucleated and non-nucleated epithelial cells Very few leucocytes
	4	Almost 100% cornified cells

In the past there has been much trouble with the appearance of quantities of nucleated epithelial cells and in some cases persistent cornification in the smears of ininjected mice. This may have been due either to lack of vitamin A or regeneration of ovarian tissue from incomplete ovariectomy. Twelve mice exhibiting this abnormality were sectioned by the Department of Pathology of Boston University School of Medicine and no ovarian tissue found. Lately, however, there has not been as much trouble. Several mice have exhibited nucleated epithelial cells, i.e a smear of 1-3, over a considerable length of time and then returned to normal. This improvement may be explained by the

fact that cod liver oil is now a part of their diet.

In testing a given preparation, preliminary smears are made for two days preceding and on the day of injection. If a new substance is to be tested, a fairly accurate impression may be obtained from experiments on three mice. Accurate standardization requires five or six mice with equal doses for each test.

In these tests, the total dose is given in three portions at four hour intervals, subcutaneously. Smears are taken at four in the afternoon of the day following injection, at nine and four on the following day, and at nine on the following day. Reaction is seldom observed in the smear before the morning of the second day, unless the preparation is unusually potent; by the third day the smear will be positive if the preparation is active.

EXPERIMENTAL RESULTS

The results of the mouse injections may be divided into five groups. These will be summarized in the following pages.

The first group consists of studies on the blood of women during various stages of the reproductive cycle. This series has been published by Janney (1929) and is summarized here with his permission.

Table I. Menstrual Cycle

Days before Catamenia	10	10-6	5-4	3-1	First day Catamenia	Second day Catamenia
Number positive	24	7	5	4	1	1
Percent positive	26	30	31	25	20	50
Number doubtful	15	5	2	0	1	0
Percent doubtful	16	22	13	0	20	0
Number negative	55	11	9	12	3	1
Percent negative	58	48	56	75	60	50
Totals	94	23	16	16	5	2

Table II. Postpartum cases

Hours postpartum	0-6	6-12	12-24	24-36	36-48	48-96	96-244	144-192
Number positive	5	1	3	4	2	1	2	0
Percent positive	83	50	37	18	8	10	33	0
Number doubtful	1	1	2	3	4	0	0	0
Percent doubtful	16	50	25	13	16	0	0	0
Number negative	0	0	3	15	19	9	4	3
Percent negative	0	0	37	68	75	90	66	100
Totals	6	2	8	22	35	10	6	3

Table III. Pregnant cases.

Lunar month of pregnancy	1	2	3	4	5	6	7	8	9	10
Number positive					2	1	6	5	14	39
Percent positive					100	33	66	83	82	95
Number doubtful					0	1	3	1	3	0
Percent doubtful					0	33	33	16	18	0
Number negative					0	1	0	0	0	2
Percent negative					0	33	0	0	0	5
Totals					2	3	9	6	17	41

From this series Janney (1929) concludes (in part) as follows:

"In a series of blood tests performed on postpartum cases, the estrus-producing substance was found to disappear rapidly after delivery from the circulating blood of the patient. This applies to the dose of hormone contained in 40 cc. of blood.

"In a series of tests performed by the same method on pregnant patients, which included 36 cases previously reported, the proportion of positive tests increased with the duration of pregnancy until it reached 95% in the tenth lunar month."

The second group of mouse tests was made on preparations made from the urine of women in the latter months of pregnancy. These extracts were prepared by B. S. Walker.

Table IV. Urine extracts.

Material	Amount	Solution in	Number of mice	Number		
				+	±	−
Crude chloroform extract	20 cc.*	Oil Sesame	2	2	0	0
	10 cc.	"	2	0	1	1
	5 cc.	"	2	2	0	0
	1 cc.	"	2	2	0	0
	4 cc.	Water	1	1	0	0
	3 cc.	"	1	0	1	0
	2 cc.	"	1	1	0	0
	1 cc.	"	1	1	0	0
Fraction soluble in cold alkali	3 mgm.	Oil Sesame	1	1	0	0
	1.5		1	1	0	0
	0.75	"	1	1	0	0
	0.38	"	2	2	0	0
	0.30	"	1	1	0	0
	0.23	"	1	1	0	0
	0.15	"	2	2	0	0
	0.075	"	1	1	0	0
	0.038	"	1	0	1	0
	0.02	"	3	1	2	0
	0.015	"	2	1	1	0
	0.008	"	3	0	1	2

* When amount is indicated in cubic centimeters, it means the equivalent of that number of cubic centimeters of the original urine.

Table IV. Urine extracts (continued)

Material	Amount	Solution in	Number of mice	Number		
				+	±	—
Fraction insoluble in cold alkali soluble in hot	1 mgm.	saline	2	1	1	0
	0.5	"	5	4	1	0
	0.1	"	2	0	2	0
	0.01	"	5	0	0	5
Preparation according to Zondek (1928)	10 cc.	"	2	2	0	0
	5	"	2	2	0	0
	1	"	2	2	0	0
	0.5	"	2	2	0	0
	0.1	"	2	1	0	1
Crystallized solid alcohol	1 mgm.	"	2	0	0	2
	0.2	"	10	0	0	10
	0.1	"	17	0	0	17
	0.01	"	2	0	0	2
Same, irradiated one half hour (quartz lamp)	1 to 0.01	"	6	0	1	5
Same, reduced	2 to 0.02	"	13	0	0	13
Mother liquor of crystals	20 cc.	"	2	1	0	1
	10	"	8	8	0	0
	5	"	8	3	4	1
	2.5	"	2	2	0	0
	1	"	2	0	2	0
	0.5	"	2	0	1	1
	0.25	"	4	0	2	2
	0.1	"	4	1	1	2
	0.01	"	2	0	1	1

Table IV. Urine extracts (continued)

Material	Amount	Solution in	Number of mice	Number +	±	−
Extracts obtained by adsorption: With aluminum hydroxide	150 cc.	Oil Sesame	4	4	0	0
	75	"	4	4	0	0
	38	"	4	2	1	1
	13	"	1	1	0	0
With animal charcoal	10	"	1	0	0	1
With barium hydrate	10	"	1	0	0	1
With barium sulfate	10	"	1	1	0	0
With nickel hydroxide	10	"	1	1	0	0
With magnesium hydroxide	10	"	1	0	0	1
Stability tests:						
Year old chloroform extract entire	2	"	6	6	0	0
Same freed from sediment	4	"	6	3	2	1
Sediment	4	"	6	0	0	6

These results will be summarized later.

A third group of experiments deal with extracts from tissues.

Table V. Tissue extracts.

Material	Amount	Solution in	Number of mice	Number +	±	—
Cat embryos about 3 weeks	3 embryos	Oil Sesame	2	2	0	0
Chick embryos 2.5 weeks	1 gram	"	4	0	2	2
Yolk sacs	1 gram	"	2	0	0	2
Placenta (formalin)	5 to 40 grams	Saline	10	0	0	10
Placenta (fresh)	40 grams	"	1	1	0	0
	20	"	1	0	1	0
	10	"	2	2	0	0
	5	"	2	2	0	0
	1	"	2	0	0	2
Liver (female)	40	"	1	1	0	0
	20	"	1	1	0	0
	10	"	1	1	0	0
	5	"	1	1	0	0

A summary of these results will appear later.

A fourth group of experiments dealt with known chemical substances, to test them for possible estrus-producing effects.

Table VI. Chemicals.

Material	Amount	Solution in	Number of mice	Number +	±	−
Oil Sesame	2 cc.	-	20	0	0	20
Oil Rape	2 cc.	-	3	0	1	2
Bile Salts	20 mgm.	Water	1	0	0	1
	10	"	2	0	0	2
	1	"	1	0	0	1
Glycocholic acid	9	"	1	0	0	1
Sodium taurocholate	5	"	2	0	0	2
	10	"	2	0	0	2
	20	"	3	0	0	3
Inosite	100	"	1	0	0	1
	50	"	6	1	0	5
	25	"	3	0	0	3
	5	"	2	0	0	2
	0.5	"	2	0	0	2

This group will be considered in a later summary.

A fifth group of experiments concerned extracts from plant sources.

Table VII. Plant extracts.

Material	Amount	Solution in	Number of mice	Number		
				+	±	−
Alder catkins (male)	160 grams	Oil Sesame	1	1	0	0
	80		3	3	0	0
	20	"	1	1	0	0
(female)	20	"	3	2	1	0
Alder leaves	30	"	6	2	1	3
	20	"	1	1	0	0
Willow catkins (male)	50	"	1	1	0	0
	20	"	5	0	0	5
(female)	20	"	5	0	1	4
	12	"	1	1	0	0
Beet juice	100 cc.	Saline	3	0	0	3
pulp	50 grams	"	3	0	0	3
	200	"	2	0	0	2
Potato juice	100 cc.	"	2	0	0	2
	50	"	2	0	0	2
	25	"	2	0	0	2
pulp	200 grams	"	2	0	0	2
	100	"	2	0	0	2
	50	"	2	0	0	2
Spinach	40	"	3	0	2	1

Table VII. Plant extracts (continued).

Material	Amount	Solution in	Number of mice	Number		
				+	±	−
Carrots	500 grams	Saline	2	0	0	2
	330	"	3	3	0	0
	250	"	4	0	1	3
	165	"	2	2	0	0
Cabbage	480	"	2	0	0	2
	330	"	3	3	0	0
	240	"	4	0	0	4
	150	"	5	3	0	2
	75	"	4	0	0	4
Apples	200	"	3	0	0	3
	100	"	3	0	0	3
Tomatoes	133	"	3	0	0	3
	67	"	3	0	0	3
Corn	500	"	2	0	0	2
	250	"	2	0	0	2
	125	"	2	0	0	2
Peaches	500	"	2	0	0	2
	250	"	4	0	0	4
Plums	500	"	2	0	0	2
	250	"	4	0	0	4
Grapes	500	"	2	0	0	2
	250	"	3	0	0	3
Sweet potatoes	500	"	2	0	0	2
	250	"	4	0	0	4

Table VII. Plant extracts (continued).

Material	Amount	Solution in	Number of mice		Number	
				+	±	−
Yeast	80 grams	Oil	3	0	2	1
	53	"	2	0	0	2
	40	"	4	0	0	4
	35	"	6	0	0	6
	20	"	3	0	0	3
Oil from oats (unsprouted)	150 grams	-	12	2	2	8
	100	-	6	0	0	6
(sprouted)	100	-	4	4	0	0
	50	-	4	4	0	0
	25	-	4	4	0	0
	20	-	2	2	0	0
	10	-	2	0	0	2
Phytin	?	Saline	3	0	0	3
Urson	10 mgm.	"	2	0	0	2
	1	"	2	0	0	2
	0.1	"	2	0	0	2
	0.01	"	2	0	0	2
Carotin, crude (amounts in equivalents of raw carrots)	2.5 pounds	Oil	1	0	1	0
	1.25	"	1	0	0	1
	0.5	"	1	0	0	1
Saponin	1 mgm.	Saline	2	0	0	2
	0.05	"	2	0	0	2

The conclusions drawn by Janney from the first series have already been quoted.

The experiments with urine extracts indicate definitely the presence of estrus-producing material in high concentration. The fraction soluble in cold alkali, although not a pure product, is potent in doses as low as .015 milligram. The greatest yield, in terms of equivalent volumes of urine is obtained with the Zondek procedure (1928). The crystallized solid alcohol, which is the same as that isolated by Marrian (1929) is entirely without action on the mouse, as was found by Marrian. Irradiation or reduction has no effect. The mother liquor from these crystallizations has the active material in it. Adsorption methods, although yielding a very pure, i.e. colorless and odorless, product, are not efficient as regards yield. The stability of chloroform solutions is shown by the positive results with year-old extracts. These extracts were kept in brown glass bottles in semi-darkness.

The experiments with tissues are not so decisive. The number of experiments with embryos is too small to permit final conclusions. They appear to contain estrus-producing material, but in low concentration. It is quite apparent that formalin destroys the activity. The positive results with extracts of fresh placenta are in agreement with the findings of other investigators. The unexpected effect of injection of liver extract remains unexplained. Gsell-Busse (1928)(1929) found estrus-producing material material in liver and bile, but not in such large amounts.

The group of chemicals tested offer mainly negative results. Oil of sesame was thoroughly tested, since it was used as a sol vent for many injections. Rape seed oil resulted in a doubtful test in one case, and was not used as a solvent. The single positive response of 50 mgm. of inosite may be considered accidental.

The group of diversified plant extracts offers results which are difficult to explain. The catkins (sexual portions) of alders appear to be active regardless of sex, while in the willow the female catkins have the higher content of estrus-producing material. The leaves,taken as controls as opposed to the sexual parts of the plants, are, however, nearly as active. The large number of fruits and vegetables tested and found negative offer little evidence to aid in any solution of the problem of the nature of the estrus-producing substance. Sprouted oats were definitely positive, while unsprouted oats were positive only with an eight-fold increase in dose. This may indicate that the presence of the active material may be associated with the growth period of the plant. It is significant from this point of view that the alder and willow catkins and leaves, which were positive, were collected in the early growing season, while the fruits and vegetables which were neagtive were fully mature when extracted. Another hypothesis which presents itself is that the active material in plants may be associated with chlorophyll. All the substances which were found positive (catkins, leaves, spinach(?) , carrots, cabbage, sprouted oats) were rich in chlorophyll, except carrots. The carotin of the carrots does not appear to be active. There is a question about the activity of carrots and cabbage since the results of different batches were quite contradictory.

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Further experiments during another growing season may help elucidate this problem.

SUMMARY.

1. Estrus in the mouse and in some other animals can be studied by means of vaginal smears.

2. Certain substances can be extracted from natural sources which will induce estrus in castrated animals.

3. The chief natural sources are: whole ovary, follicular fluid, placenta, urine from women in the latter months of pregnancy.

4. The chief method of extraction is with the fat solvents.

5. The material can be partially purified by various methods; whether or not it has been definitely isolated is still a matter of question.

6. Chemically it is characterized by stability to heat and solubility in fat solvents. It appears to contain only carbon, hydrogen and oxygen.

7. The only method of quantitative measurement is by biological assay, preferably by the vaginal smear method.

8. Associated with its property of producing estrus, this material appears to exert a stimulating effect on the reproductive system of the female; some of the observed effect may be due to other hormones, however.

9. Detailed description is given of the methods used by the writer in maintaining a colony of mice for test purposes, and in carrying out the tests.

10. Results are given of 457 mouse tests, made on extracts of urine, tissues, chemicals and plant extracts.

11. The significance of some of these results is briefly discussed.

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